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rap1B, a cAMP-dependent Protein Kinase Substrate, Associates with the Platelet Cytoskeleton*

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rap1B is a member of the *ras* superfamily of low molecular weight GTP binding proteins which constitutes a focal point of GTP and cAMP signal transduction systems. Like other members of this superfamily, rap1B is membrane-associated in resting platelets, presumably through polyisoprenylation. The studies presented here were undertaken to determine the subcellular changes in rap1B localization during cell activation. Activated and unactivated platelets were fractionated by Triton X-100 lysis followed by differential centrifugation to obtain a 10,000 × *g* cytoskeleton fraction, a 100,000 × *g* membrane skeleton fraction, and a 100,000 × *g* supernatant fraction containing solubilized proteins. In unactivated platelets, rap1B was present in the 100,000 × *g* supernatant fraction. In contrast, in platelets activated with 1 unit/ml α-thrombin or with the calcium ionophore, A23187, rap1B was quantitatively recovered in the 10,000 × *g* cytoskeleton fraction. rap1B was absent from the 100,000 × *g* fraction containing the membrane skeleton and could not be detected in the 100,000 × *g* supernatant containing cytosolic proteins and solubilized membrane components. These results indicate that rap1B associates with the cytoskeleton during cell activation.

Low molecular weight GTP-binding proteins are ubiquitous cellular components which function as molecular switches early in the signal transduction pathways for cell growth and differentiation (1–3). Control of this switch is through guanine nucleotide binding; GDP-ligated forms of these proteins are inactive while GTP-ligated forms are active, stimulating cell growth and differentiation. According to current views, following an extracellular signal for activation, the inactive GDP-ligated protein is converted to the active GTP-ligated protein through a nucleotide exchange reaction. Conversion back to the inactive GDP-ligated protein is facilitated by cytosolic proteins known as GTPase-activating proteins, which enhance the endogenous GTPase activity of the low molecular weight proteins (4). To date, more than 20 distinct low molecular weight G proteins[1] have been identified (5). The prototype for these proteins is $p21^{ras}$, the product of the *ras* protooncogene.

The subcellular localization of the low molecular weight G proteins appears to be essential for their activity. Two sites of membrane localization have been identified. $p21^{ras}$ and its homologs are localized to the cytoplasmic face of the plasma membrane, and this localization is absolutely required for transforming activity (6). Localization to the plasma membrane is a complex process that involves a series of posttranslational modifications resulting in covalent attachment of a polyisoprenyl group, typically farnesyl, to the C-terminal cysteine residue (7). The signal for polyisoprenylation is the C-terminal propeptide sequence, Cys-Ali-Ali-Xaa (where Ali is any aliphatic amino acid and Xaa is any amino acid). Following polyisoprenylation, the three C-terminal amino acids (Ali-Ali-Xaa) are proteolytically removed and the C-terminal cysteine is carboxymethylated to yield the mature protein with a polyisoprenylated C-terminal cysteine residue. Mutations of the C-terminal cysteine residue block both polyisoprenylation and membrane localization, as well as the transforming activity (6, 8). The localization of these proteins to the plasma membrane is presumed to be essential for their signal-transducing activity. Another group of low molecular weight G proteins, represented by the yeast proteins *Sec4* and *YPT1*, have been localized to internal membranes (9, 10). These proteins lack the Cys-Ali-Ali-Xaa signal required for polyisoprenylation but have a C-terminal cysteine residue. It is not known if these proteins are polyisoprenylated or palmitylated.

Recently, a 22-kDa substrate for cAMP-dependent protein kinase in human blood platelets, previously termed p22 or thrombolamban (11–16), was identified as a low molecular weight G protein. This identification was based on evidence that the protein hydrolyzed GTP and was recognized by antibodies against the GTP-binding domain of $p21^{ras}$ (17–21). Partial protein sequence for the platelet protein (22–24)[2] was identical with rap1B (26), a member of the *ras* superfamily also known as smg-p21B (17) or m22KG(I) (18). Like $p21^{ras}$, rap1B in platelets is membrane-associated (19, 21) and has a consensus polyisoprenylation sequence (23–24).[2] Rap1B is phosphorylated in intact platelets by prostaglandin I_2 or lipid soluble analogs of cAMP and in membrane fractions by type I cAMP-dependent protein kinase or the catalytic subunit of cAMP-dependent protein kinase (11–16, 20). The phosphorylated amino acid was shown to be a serine (16), and the sequence at the phosphorylation site has been identified as -Arg-Lys-Lys-Ser-Ser-Cys-COOH (24).[2] Phosphorylation of rap1B in intact platelets results in translocation of the protein from a membrane fraction to a cytosolic fraction (19). Phos-

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[1] The abbreviations used are: G protein, GTP-binding protein; MES, 4-morpholineethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

[2] T. H. Fischer, J. H. Collins, M. Gatling, and G. C. White II, *FEBS Lett.*, submitted for publication.

phorylation had no effect on the GTPase activity of rap1B, and the phosphorylation of GTP- and GDP-bound forms of rap1B was kinetically similar (17).

Because of its potential importance in cell activation and its known membrane localization, the present study was undertaken to examine the cellular localization of rap1B in platelets during activation and was based on several observations. First, since rap1B is membrane-associated, probably through either palmitylation or polyisoprenylation of the C-terminal cysteine residue, it was important to determine what happens to the protein during normal cell function. Second, the site of phosphorylation of rap1B is remarkably close to the site of membrane attachment (24),[2] suggesting that membrane attachment may be a regulatable event. Third, several laboratories have reported that palmitylated proteins, including unidentified proteins in the 18–24-kDa range, are incorporated into the platelet cytoskeleton when platelets are activated (27). Finally, recent evidence suggests that some members of the *ras* family of proteins can be associated with intracellular membrane fractions, such as the Golgi apparatus and secretory vesicles (9, 10). The results obtained here demonstrate that rap1B associates with the cell cytoskeleton during platelet activation and suggest a possible role for this cytoskeleton interaction in platelet function.

EXPERIMENTAL PROCEDURES

Reagents, buffers, and salts were obtained from Sigma. M90, a murine monoclonal antibody against $p21^{Hras}$ was produced as described previously (28). Human α-thrombin, kindly provided by Dr. Frank Church (Chapel Hill, NC), was prepared as detailed elsewhere (29) and was stored at −80 °C in 750 mM NaCl, 20 mM MES, 3 mM NaN_3, pH 7.5, until use.

Platelets were prepared by differential centrifugation from fresh human blood as described previously (30) and suspended at a concentration of 10^9 cells/ml in citrated saline. Platelets were aggregated with stirring by the addition of $CaCl_2$ to a final concentration of 5 mM followed 1 min later by 1/100 dilution of stock thrombin to a final concentration of 1 unit/ml, A23187 to a final concentration of 20 μM, or control buffer. After 5 min, cytoskeletons were extracted by a Triton X-100 lysis procedure (31). An equal volume of "lysis buffer" (145 mM NaCl, 0.1 mM $MgCl_2$, 15 mM HEPES, 1.0 mM phenylmethylsulfonyl fluoride, 10 mM EGTA, 0.8 μg/ml leupeptin, 1% (w/v) Triton X-100, pH 7.4) was added. After 5 min at 0 °C, the samples were centrifuged at 10,000 × *g* for 5 min. The resulting pellet was resuspended in "modified lysis buffer" (145 mM NaCl, 0.1 mM $MgCl_2$, 15 mM HEPES, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM EGTA, 0.4 μg/ml leupeptin, 1% (w/v) Triton X-100, pH 7.4) and centrifuged again as in the last step to obtain the cytoskeletal pellet. The 10,000 × *g* supernatant from the initial centrifugation step was centrifuged at 100,000 × *g* for 1 h to obtain a 100,000 × *g* pellet, which contained the membrane skeleton (32), and a corresponding 100,000 × *g* supernatant that contained soluble cytosolic proteins and Triton X-100-solubilized membrane components.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (33) with reducing conditions on 12.5% polyacrylamide gels. Molecular mass standards were soybean trypsin inhibitor (21 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (67 kDa), phosphorylase *b* (97 kDa), and myosin (200 kDa). The amount of protein applied to each electrophoretic lane depicting intact platelets, 10,000 × *g* pellets, and 100,000 × *g* pellets was that obtained from 10^8 platelets, while the supernatant phases were from 2.5 × 10^8 cells/lane. For Western blot analysis with the monoclonal antibody M90, proteins were electrotransferred to nitrocellulose and then immunodetected with a variation of the procedure of Towbin *et al.* (34) that used an alkaline phosphatase-conjugated second antibody system as detailed elsewhere (21).

RESULTS

To determine if rap1B was a component of the activation-dependent cytoskeleton, resting platelets and platelets activated by the addition of 1 unit/ml α-thrombin were lysed in "lysis buffer" and then fractionated by differential centrifugation to recover a 10,000 × *g* pellet containing the activation-dependent cytoskeleton, a 100,000 × *g* pellet containing the membrane skeleton, and a 100,000 × *g* supernatant containing solubilized membrane components and cytosolic proteins (31, 32). The results are presented in Fig. 1. In control platelets (*panel A*), rap1B was present in the 100,000 × *g* supernatant (*lanes 7* and *8*) and was absent from the cytoskeleton (*lanes 3* and *4*) and the membrane skeleton (*lanes 5* and *6*). As previously reported, the activation-dependent cytoskeleton is not fully assembled in resting platelets and contains mostly actin (31). Upon activation (*panel B*), there were marked changes in the composition of the cytoskeletal fraction. On Coomassie Blue-stained gels, protein bands with apparent molecular masses of 250, 200, 92, and 44 kDa, corresponding

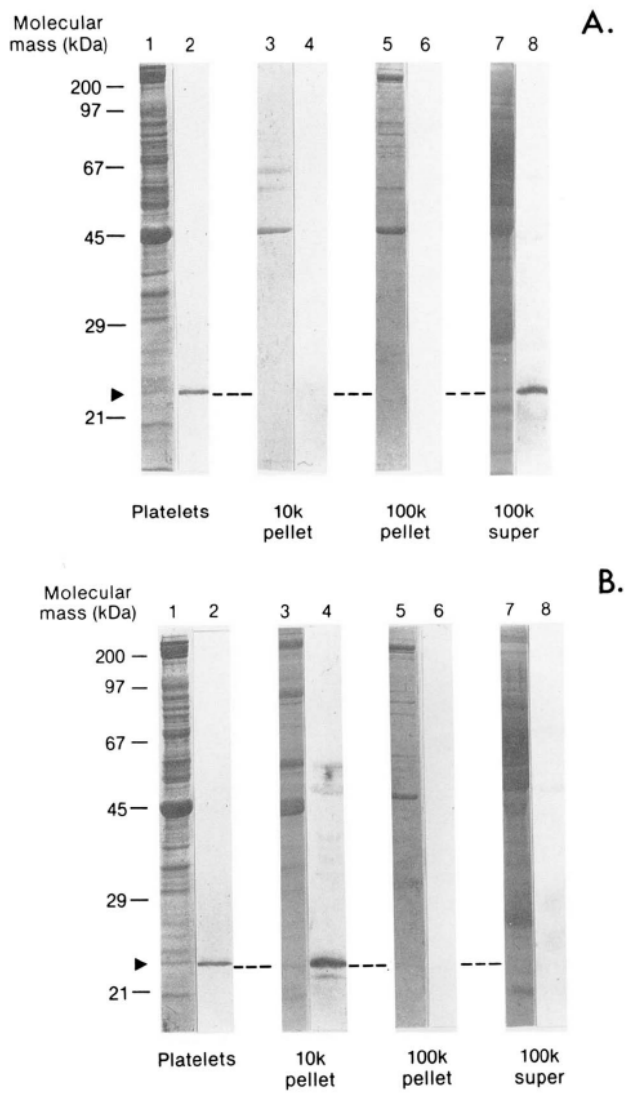

FIG. 1. **Cytoskeletal association of rap1B.** Washed human platelets were recalcified and aggregated by the addition of buffer (*panel A*) or 1 unit/ml human α-thrombin (*panel B*). After 5 min, cytoskeletal structures were isolated by Triton X-100 lysis and differential centrifugation. In each *panel*, *lanes 1*, *3*, *5*, and *7* are Coomassie Blue-stained gels and *lanes 2*, *4*, *6*, and *8* are Western blots developed with the murine monoclonal antibody, M90. *Lanes 1* and *2* are intact platelets (*platelets*), *lanes 3* and *4* are the cytoskeletal fraction (*10k pellet*), *lanes 5* and *6* are the membrane skeleton fraction (*100k pellet*), and *lanes 7* and *8* are solubilized platelet proteins (*100k super*). The *arrow* and *dashed lines* refer to the position of rap1B. Molecular mass standards are shown on the *left* in each *panel*. This result is representative of four experiments.

to the known cytoskeletal components actin-binding protein, myosin, α-actinin, and actin, respectively, were observed in the cytoskeletal fraction (*lane 3*). Protein bands were also seen at 55–60 kDa, corresponding to the α, β, and γ chains of fibrinogen, and at 22 kDa. On Western blots, rap1B was found entirely in the cytoskeletal fraction (*lane 4*) and had the same electrophoretic mobility as the 22-kDa band seen in Coomassie-stained lanes. Fractions containing the membrane skeleton (*panel B lanes 5* and *6*) or the supernatant (*lanes 7* and *8*) did not contain detectable rap1B by either Western blot with M90 or by protein stain. When platelets were activated with the calcium ionophore A23187, results similar to those depicted in Fig. 1 were obtained (data not shown).

DISCUSSION

We present evidence that rap1B, a *ras*-like protein that both binds GTP and is a substrate for cAMP-dependent protein kinase, is incorporated into the cytoskeleton of human platelets during cell activation. These data provide direct evidence for a possible role of rap1B in cytoskeletal assembly and amplify previous studies in other cells indirectly implicating low molecular weight G proteins in cytoskeletal function (35, 36).

The cellular skeleton of platelets contains several structural components (31, 32, 37). The membrane skeleton consists of short actin filaments cross-linked by actin-binding protein and is linked to the glycoprotein Ib-IX complex in the plasma membrane. It provides the platelet with its shape and plays an important role in determining the organization and function of the GPIb-IX complex in the plasma membrane. This structure is present in resting platelets but is disrupted following platelet activation, at least in part due to the cleavage of actin-binding protein by the calcium-activated protease (38, 39), calpain, resulting in changes in platelet shape and increased mobility of membrane proteins. The cytoplasmic skeleton or cytoskeleton from resting platelets consists primarily of a mesh of actin filaments. When platelets are activated, actin, myosin, and actin-binding protein are incorporated into the cytoskeleton. Our results indicate that rap1B is a component of the activation-dependent cytoskeleton, but not of the membrane skeleton, even though rap1B is membrane-bound in resting platelets. Furthermore, in contrast to other cytoskeletal proteins such as actin-binding protein and actin, which are only partly incorporated into the cytoskeleton, all of the immunodetectable rap1B becomes incorporated into the cytoskeleton during activation. This quantitative incorporation of rap1B is distinct and suggests that the cytoskeletal interaction is an important one.

In addition to rap1B, two other cytoskeletal proteins have been identified as cAMP-dependent protein kinase substrates: the β subunit of GPIb (40), which is present in the membrane skeleton, and actin-binding protein (41). Cyclic AMP-dependent phosphorylation of GPIbβ in intact platelets did not inhibit agonist-stimulated aggregation, secretion, or phosphorylation of myosin light chain or p47. However, agonist-stimulated actin polymerization was inhibited suggesting that phosphorylation of GPBIbβ influenced cytoskeletal assembly (25). In preliminary studies, we have tried to examine the effect of phosphorylation on rap1B incorporation into the cytoskeleton, but treatment with agents that increased cAMP levels inhibited cytoskeletal assembly.[3] The identification of three proteins involved in the cAMP pathway as components of the platelet cytoskeleton is remarkable and suggests that at least one of the inhibitory mechanisms of cAMP is through an effect on the cytoskeleton.

[3] T. H. Fischer and G. C. White II, unpublished data.

The function of rap1B and other low molecular weight G-proteins in platelets remains unclear. Our demonstration that rap1B incorporates into the cytoskeleton during cell activation suggests that it functions less in initial signal transduction and more in subsequent events. Indeed, if the proposed roles of *Sec4* and *YPT1* in yeast (9, 10) can be extended to other systems, one might speculate that rap1B acts as a targeting mechanism in the cytoskeleton, perhaps mediating protein-protein interactions or the interaction of the cytoskeleton with another cellular component, such as secretory granules.

In summary, the membrane interactions of rap1B in platelets appear to be complex but may ultimately yield important clues to the function of the low molecular G proteins. In resting platelets, rap1B is associated with the cell membrane, probably the plasma membrane, and probably through a polyisoprenyl group. Whether or not this involves changes in the posttranslational modification of rap1B remains to be determined. When the cell is inhibited by cAMP, rap1B is translocated from the membrane to a cytosolic location (19). When the cell is activated, rap1B becomes incorporated into the cytoskeleton. This bidirectional translocation may be essential for normal cell function.

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